Rosmarinic acid, major phenolic constituent of greek sage herbal tea, modulates rat intestinal SGLT1 levels with effects on blood glucose

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Abbreviations: BBM, brush-border membrane; **GLP-1,** glucagon-like peptide 1; **GLUT2,** facilitative glucose transporter 2; **HC,** high carbohydrate; **Hsp70,** heat shock protein 70; **LC,** low carbohydrate; **PKC,** protein kinase C; **RA,** rosmarinic acid; **SFT,** *Salvia fruticosa* tea; **SGLT1,** Na(+)-glucose cotransporter 1; **STZ,** streptozotocin; **T2DM,** type 2 diabetes mellitus.

Keywords: Diabetes mellitus; Intestinal glucose absorption; Rosmarinic acid; *Salvia fruticosa* Miller; SGLT1 expression;

Abstract

Scope: Previous results suggested the effects of *Salvia fruticosa* tea (SFT) drinking on glucose regulation might be at the intestinal level. Here, we aim to characterize the effects of SFT-treatment and of its main phenolic constituent – rosmarinic acid (RA) – on the levels and localization of the intestinal Na+/glucose cotransporter-1 (SGLT1), the facilitative glucose transporter 2 (GLUT2) and glucagon-like peptide-1 (GLP-1).

Methods and results: Two models of SGLT1 induction in rats were used: through diabetes induction with streptozotocin (STZ) and through dietary carbohydrate manipulation. Drinking water was replaced with SFT or RA and blood parameters, liver glycogen and the levels of different proteins in enterocytes quantified. Two weeks of SFT treatment stabilized fasting blood glucose levels in STZ-diabetic animals. The increase in SGLT1 localized to the enterocyte brush-border-membrane (BBM) induced by STZ treatment was significantly abrogated by treatment with SFT, without significant changes in total cellular transporter protein levels. No effects were observed on GLUT2, Na^+/K^+-ATPase or GLP-1 levels by SFT. Additionally, SFT and RA for 4 days significantly inhibited the carbohydrate-induced adaptive increase of SGLT1 in BBM.

Conclusion: SFT and RA modulate the trafficking of SGLT1 to the BBM and may contribute to the control of plasma glucose.

1 Introduction

Diabetes mellitus affects 10 to 20% of adults worldwide and is characterized by high levels of glucose in the blood. Particularly type 2 diabetes mellitus (T2DM), that corresponds to 90 to 95% of all cases of diabetes, is attaining epidemic proportions in populations with western-type diets and lifestyles [1], in part due to loss of traditional cooking with local ingredients, including herbs and spices. This justifies the search for new active principles and therapeutical targets that help prevent and limit the progression of this disease. Plants with antidiabetic reputation may be good sources of compounds with these properties.

Herbs, such as ginseng (*Panax* spp.), fenugreek (*Trigonella foenum graecum*) and bitter melon (*Momordica charantia*) and spices, such as cinnamon (*Cinnamomum zeylanicum*), are examples of plants where antidiabetic properties have been identified in experimental animals and clinical trials with T2DM patients [2-5]. *Salvia* species, such as *Salvia officinalis* and *Salvia fruticosa*, have also been used for their antidiabetic properties [6, 7]. Because these plants are edible and palatable, they produce their effects by inclusion in the diet. However, active principles and mechanisms of action have not been elucidated.

Glucose released upon carbohydrate digestion is absorbed mainly in the jejunum of the small intestine. The Na (+)-glucose cotransporter (SGLT1), localized to the enterocyte apical or brush-border membrane (BBM), and the basolateral facilitative glucose transporter 2 (GLUT2) are the main intestinal sugar transporters responsible for glucose uptake from the intestinal lumen into the blood [8]. The Na^+ gradient necessary for SGLT1 activity is maintained by the basolateral Na^+/K^+-ATPase.

The intestine has the ability to adapt functionally as well as morphologically to stimuli such as diet composition and disease processes, such as diabetes [9]. Induction

of SGLT1 expression in the BBM of enterocytes is produced by ingesting diets rich in carbohydrates that increase available luminal glucose [10]. In addition, an increase in SGLT1 expression in jejunal enterocytes is associated with diabetes in humans (4.3-fold in BBM [11]) and experimental animals, resulting in increased monosaccharide absorption [12]. GLUT2 levels have also been shown to be increased in the intestine of both diabetic rats and human patients [11, 12]. These changes combined with intestinal hyperplasia increase the capacity for glucose absorption in diabetic individuals, which aggravates undesirable postprandial hyperglycemia. Therefore, strategies that delay digestion and absorption of intestinal glucose are beneficial in a diabetes scenario. The delay of digestion is a therapeutic strategy currently addressed by the oral antidiabetic drug acarbose, an alpha-amylase and alpha-glucosidase inhibitor [13].

Also contributing to the diabetic postprandial hyperglycemia is a reduction in the incretin effect due to loss of insulin response to gastric inhibitory polypeptide and to a significantly reduced secretion of glucagon-like peptide 1 (GLP-1) [14]. New strategies to prevent the loss of GLP-1 producing cells would also be beneficial.

The present study aims at characterizing the effects of greek sage (*Salvia fruticosa* Mill.) tea and rosmarinic acid (RA; corresponding to 72% of the total phenolics present in this tea) on intestinal expression of glucose transporters (SGLT1 and GLUT2) and Na^+/K^+-ATPase in response to streptozotocin-induced diabetes and dietary carbohydrates. In view of the involvement of Hsp70 and PKC on the SGLT1 activity and vesicle trafficking to the BBM, effects on the expression levels of these proteins were also determined. Effects on GLP-1 and pancreatic islet regeneration were evaluated as well.

2 Materials and methods

2.1 Plant material and preparation of S. fruticosa water extract

Salvia fruticosa plants were cultivated in an experimental farm located in Merelim, Braga, Portugal, and were collected in June 2004. The aerial parts of plants were air dried and kept a -20ºC with the accession number SF062004, under the responsibility of the Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Minho. Voucher specimen is also kept in an active bank in Braga, Portugal, also under the responsibility of the CITAB.

Since sage is traditionally used as a tea, a water extract of *S. fruticosa* was routinely prepared as previously described [15] by pouring 150ml of boiling water onto 2g of the dried plant material and allowing to steep for 5 min. The preparation produced a 2.8±0.1mg of extract dry weight per ml of infusion (0.28% w/v) and a yield of 19.1% (w/w) in terms of initial crude plant material of *S. fruticosa*. Sub-samples of freeze-dried extract (0.01g) were redissolved in 1ml of ultrapure Milli Q water and aliquots of 20µl were injected into the HPLC/DAD system and analyzed by HPLC/DAD as previously described [16]. Rosmarinic acid (577.29µg/ml), 6-hydroxyluteolin-7-glucoside (104.78µg/ml) and a heteroside of an unidentified flavone (99.13µg/ml) were the most representative phenolic compounds.

2.2 Animals

Male Wistar rats (6 weeks) were purchased from Charles River Laboratories (Barcelona, Spain) and kept in the authorized animal facilities of the Life and Health Sciences Research Institute (ICVS) from University of Minho. The animals were maintained under controlled temperature (20±2ºC) and humidity (55±10%) with a 12 h light:12 h dark cycle, and given food and tap water *ad libitum*. Animals were kept and handled in accordance the NIH guidelines for the experimental use and care of

laboratory animals by authorized investigators by the National Veterinary Agency (DGV: Direcção Geral de Veterinária), Portugal, and the experiment approved by the university's ethics committee.

Diabetes was induced by a single i.p. injection of freshly prepared streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA) solution (45mg/Kg in 0.1M acetate buffer, pH 4.5 given in a volume of 1ml/Kg body weight [17]) to overnight-fasted rats. Control animals received a sham injection with buffer only. Diabetes was identified by polydipsia, polyuria and by measuring non-fasting plasma glucose levels 48 h after injection of STZ. One week after STZ injection, rats with fasting blood glucose levels of 250-350mg/dl were used in the experiment.

2.3 Experimental design

2.3.1 Diabetic animals induced by STZ were used as a model of SGLT1 induction in intestinal mucosa. Twenty four rats were used and divided into four groups (two healthy and two STZ-diabetic) of six rats each. Animal groups were subjected to the following treatments for 14 days (Fig. 1A): group 1 – healthy rats drinking water; group 2 – healthy rats drinking *S. fruticosa* tea (SFT); group 3 – STZ-induced diabetic rats drinking water; group 4 – STZ-induced diabetic rats drinking SFT. Water/SFT (made in tap water) and food were given *ad libitum* and the beverage was renewed daily. Due to the diabetic-induced polydipsia, SFT was diluted in group 4 in order to give the same dose as in group 2, based on the beverage consumption measured in the previous day. The replacement of water by SFT did not change food and beverage consumption, or animal body weight increase.

At the end of the treatment, 16 h-fasted animals were sacrificed by decapitation and the intestinal mucosa (40cm of jejunum) scraped off on ice with a glass microscope slide,

after washing with PBS pH 7.4 (containing 40mM PMSF in ethanol added fresh). The intestinal mucosa was immediately frozen in liquid nitrogen and stored at -80ºC until use. Prior to scraping a small piece of intact jejunum (from the middle of the above 40cm region) was collected as well as pancreatic tissue for fixation in 4% paraformaldehyde in PBS pH 7.4, for 24 h at 4ºC. The tissues were then stored in ethanol 70% (v/v) until being processed for paraffin embedding. Blood samples were also collected to measure glucose and insulin levels, as well as the activity of liver transaminases.

2.3.2 An additional model of SGLT1 induction in intestinal mucosa through dietary carbohydrate manipulation was used. For that, thirty rats were used and divided into five groups of six animals each, where (Fig. 1B): group 1 – rats were fed with water and food (normal rat chow – referred as high carbohydrate diet – HC) *ad libitum* for 14 days; group 2 – rats were fed with water and food (HC) *ad libitum* for 7 days, and afterwards fed for 7 days with a soybean diet (low carbohydrate diet – LC) replacing the normal rat chow *ad libitum*; group 3 – rats were treated as in group 2, and afterwards fed for 4 days more with water and HC diet *ad libitum*; group 4 – rats were treated as in group 2, and afterwards fed for 4 days more with daily fresh *S. fruticosa* tea (replacing the water drinking) and HC diet *ad libitum*; group 5 – rats were treated as in group 4, with daily fresh RA solution replacing the *S. fruticosa* tea drinking. The RA solution was prepared in tap water diluting RA (Sigma-Aldrich) to the same concentration found in the SFT (577µg/ml).

The composition of the food given to the rats are presented in Supporting Information Table 1, where the normal rat chow (UAR-A04 chow diet, Reus, Spain) with 60.3% carbohydrates was considered the HC diet and the soybean diet with 28.0% of carbohydrates was referred as LC diet (Soybean meal 47.5, Cargill S.A.C.I., Buenos

Aires, Argentina, kindly supplied by NANTA, Fábricas de Moagem do Marco S.A., Marco de Canaveses, Portugal).

Animals were sacrificed by decapitation and the intestinal mucosa (40cm of jejunum) was scraped off and stored as in the previous experiment. Blood samples were also collected to measure plasma glucose levels.

2.4 Isolation of Brush-Border Membranes

Brush-Border Membranes (BBM) were isolated from frozen jejunal mucosal scrapings using a combination of cation precipitation and differential centrifugation as described previously [18] with few modifications (for details, see Supporting Information *Materials and methods*). BBM were then frozen in liquid nitrogen and stored at -80ºC until use. The enrichment of the brush-border marker (by measuring the activity of alkaline phosphatase [19]) was about 10 times the mucosa crude homogenate. Protein content was measured with the Bradford Reagent (Sigma-Aldrich) using BSA as a standard.

2.5 Western blotting

The levels of SGLT1, GLUT2 and Hsp70 in the BBM was quantified by Western blotting as described previously [20, 21]. BBM protein were solubilized in Laemmli's buffer [22] and heated for 15 min at 70ºC. Then, 25µg of protein were loaded in each well and separated by SDS-PAGE and transferred onto Hybond-P polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK). Membranes were blocked in 5% (w/v) non-fat dry milk in TPBS (0.05% (v/v) Tween 20 in PBS) pH 7.4, for 1 h, at room temperature and then incubated with rabbit antibody to rat SGLT1 (raised in rabbits against a peptide comprising amino acids 582-600:

EEDPKDTIEIDAEAPQKEK of rat SGLT1 [23]) or rabbit polyclonal to SGLT1 (Abcam, Cambridge, UK) diluted 1:500 or 1:2,000, respectively, overnight at 4ºC. After secondary antibody incubation, immunoreactive bands were detected by chemiluminescence exposing to a film. Membranes were also probed against GLUT2 (using rabbit polyclonal antibody from Chemicon International, Temecula, CA, USA) and Hsp70 (using a mouse monoclonal antibody from Sigma-Aldrich).

The same procedure was used to quantify the abundance of SGLT1, GLUT2, Hsp70, Na^+/K^+-ATPase (using α5 mouse monoclonal antibody [24]) and PKC (using a rabbit polyclonal antibody; Chemicon International, Temecula, CA, USA) in whole cell homogenates of jejunal mucosa. The Na^+/K^+-ATPase antibody was obtained as culture supernatant from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, under contract N01-HD-7-3263 from The National Institute of Child Health and Human Development.

The intensity of the immunoreactive bands was measured from digitized images, calibrated with a Kodak grey scale, using Sigma Scan Pro (v5) software (SPSS, Chicago, IL, USA), or acquired by a chemiluminescence detection system, the Chemi Doc XRS (BioRad Laboratories, Inc., Hercules, CA, USA), being the band area intensity quantified using the Quantity One software from BioRad. Results are presented as percentage of band area intensity of controls.

2.6 Glucose measurement

In the experiment of STZ-diabetic rats, blood glucose levels from overnight fasted animals were monitored with the Accutrend® GCT device (Roche diagnostics GmbH, Mannheim, Germany) using Accutrend® test strips for glucose (Roche diagnostics GmbH), during the experimental period.

In the diet manipulation experiment, plasma glucose levels from *ad libitum* fed animals were measured using a colorimetric enzymatic method – Glucofix (A. Menarini Diagnostics, Firenze, Italy) – following manufacturer specifications.

2.7 Insulin measurement

The content of insulin in rat plasma was measured using an ELISA-based commercial kit – Rat Insulin EIA Kit (SPI-BIO, Montigny-le-Bretonneux, France) – following the manufacturer specifications.

2.8 Liver glycogen content

Liver glycogen content was quantified with amyloglucosidase as previously described [25]. Dilutions of the liver homogenate were used to ensure that the determination was done within the linear phase. The glycogen content was expressed in μmol glucose per g of liver.

2.9 Statistical Analysis

Data are expressed as means with standard errors of the means (SEM).

For statistical analysis a two-way ANOVA was employed followed by the Newman-Keuls multiple comparison test (SigmaStat, version 2.03; SPSS Inc., San Rafael, CA, USA) to compare physiological conditions (healthy vs. diabetic) and the effect of *in vivo* beverage (water vs. SFT). P values ≤ 0.05 were considered statistically significant. Student *t*-tests were used to compare differences within the different carbohydrate groups, namely HC-HC, HC-LC and HC-LC-HC/W groups. Then, to compare the effects of different drinking regimes (water-W; *S. fruticosa* tea-T; and, rosmarinic acid-RA) within the HC-LC-HC carbohydrate groups, a one-way ANOVA followed by the

Newman-Keuls multiple comparison test were employed. P values ≤ 0.05 were considered statistically significant.

3 Results

3.1 SFT treatment significantly improves diabetic fasting blood glucose levels, but has no effects on plasma insulin and liver glycogen content

As expected, one week after the i.p. injection of STZ, fasting blood glucose increased from about 119 ± 4 mg/dl in healthy rats to 311 ± 25 mg/dl in STZ-induced diabetic animals (Fig. 2A). Plasma insulin concentration was significantly lower in diabetic versus non-diabetic animals (Fig. 2B). In water drinking STZ-diabetic animals plasma glucose levels continued to increase throughout the 14 day experimental period but remained stable in SFT drinking STZ-diabetic animals (Fig. 2A). On day fourteen, SFT-treated diabetic animals showed significantly lower blood glucose levels than water drinking controls (Fig. 2A). In non-diabetic (healthy) animals, fasting glucose levels remained constant and were not affected by SFT drinking.

SFT drinking did not significantly changed liver glycogen content (Fig. 2C) or induced liver toxicity (monitored by plasma transaminase levels – (Supporting Information Table 2) or increased regeneration of beta-cell mass (Supporting Information Fig. 1).

3.2 SFT treatment decreases enterocyte BBM SGLT1 in diabetic animals but has no effects on healthy animals

As shown in Fig. 3, STZ-diabetic rats showed a significant increase in SGLT1 levels both in whole cell homogenates (40%) and in BBM (85%), when compared with controls. Treatment with SFT for 14 days did not change SGLT1 levels in whole cell homogenates (Fig. 3A), but limited the increase in BBM to about 30% of levels in

untreated controls (Fig. 3B). In healthy animals sage tea drinking did not change SGLT1 levels.

In addition to its presence on the basolateral membrane, GLUT2, alongside SGLT1, may play a role in intestinal glucose absorption at the BBM [11, 12, 26], where its abundance has been shown to increase with diabetes induction [26]. The levels of GLUT2 were significantly increased in whole cell homogenates of jejunal mucosa of STZ-diabetic rats when compared with healthy controls (Fig. 4A) but not in BBM (Supporting Information Fig. 2). Levels of Na^+/K^+-ATPase were higher in diabetic animals (Fig. 4B) and the number of GLP-1 expressing cells per cm of villus, determined by immunohistochemistry (Supporting Information Fig. 3), was smaller in diabetic animals compared with healthy controls. All these parameters were not affected by SFT treatment.

3.3 SFT and particularly rosmarinic acid decrease fed state plasma glucose levels in animals fed a high carbohydrate diet

As shown in Fig. 5, feeding the LC diet for 7 days (HC-LC group) produced a significant 15% reduction in plasma glucose levels (in fed animals), when compared with HC-HC group. Four days after the reintroduction of the HC diet (HC-LC-HC group), plasma glucose returned to control levels. However, in the RA group, the return of plasma glucose to control levels was completely inhibited (Fig. 5).

3.4 SFT and rosmarinic acid significantly decrease SGLT1 in BBM but not total enterocyte SGLT1 levels in high carbohydrate fed animals

SGLT1 levels in the BBM of enterocytes increase with increasing digestible dietary carbohydrates and can be down regulated by removing carbohydrate from the diet. Four

days (but not 2; see Supporting Information Fig. 4) after the reintroduction of a high carbohydrate diet (HC) to animals where enterocyte BBM and total SGLT1 levels had been dramatically reduced (by feeding a low carbohydrate (LC) diet for 7 days) resulted in complete recovery of SGLT1 levels (Fig. 6). Replacing water with SFT or RA upon reintroduction of the HC diet caused a significant inhibition of the adaptive increase of SGLT1 levels in BBM of about 32% and 50%, respectively (Fig. 6B), without affecting total levels (Fig. 6A).

3.5 SFT and rosmarinic acid decrease BBM Hsp70 and PKC levels

As shown in Fig. 7, the levels of the constitutive form of Hsp70 in BBM, which corresponds to only a small part of total Hsp70 (Fig. 7C), decreased significantly in LC fed animals, but returned to normal levels 4 days after the reintroduction of HC diet. SFT and RA treatments inhibited this recovery, the effect being significant for RA. The levels of Hsp70 in whole cell homogenates were not changed among the different groups (Supporting Information Fig. 5). PKC levels (Fig. 8) were not affected by diet, but were significantly lower in RA-treated animals.

4. Discussion

Control over carbohydrate digestion and absorption is beneficial in the management of diabetes since it helps contain postprandial hyperglycemia excursions thereby improving glycemic control and reducing the risk of diabetic complications [27]. Many studies have reported that diabetes enhances intestinal glucose absorption, although the mechanisms that underlie this effect are poorly understood. Higher levels of intestinal BBM glucose transporters in both diabetic patients and experimental diabetic animals [11, 12, 26, 28], contributes significantly to the increased glucose

absorption in diabetes. However, there are, to our knowledge, no therapeutic attempts to limit it. Inhibitors of SGLT1, such as phloridzin, have been known, although its degradation by intestinal lactase-phloridzin hydrolase has been pointed out to limit its pharmacological value. Inhibition of SGLT1 activity by other natural compounds, such as tea polyphenols (e.g. epigallocatechin gallate) has also been shown [29]. However, few studies show effects on SGLT1 levels in the BBM, where it is functionally active. Recently, Gum Arabic has been shown to produce this effect [30]. Also, Miró-Queralt *et al.* [31] also reported that sodium tungstate normalized SGLT1 expression in the jejunum of STZ-diabetic rats.

Fourteen days of treatment with SFT presented no toxicity (plasma transaminases not changed) and prevented further deterioration of glucose homeostasis in STZ diabetic rats without affecting plasma insulin levels or liver glycogen deposition. In a previous report [6], SF water extracts was shown to decrease plasma glucose after an oral glucose tolerance test but not an intravenous glucose tolerance test, suggesting SFT acts at the intestinal level rather than by improving peripheral insulin sensitivity.

Because the intestinal effects are not due to digestive enzyme inhibition (unpublished observations), in the present study, we hypothesized that SFT would exert control over blood glucose through modulation of enterocyte glucose transporter levels. To test this, two models of SGLT1 induction in rats were used: diabetes induction with STZ and dietary carbohydrate manipulation.

As expected, three weeks after STZ administration to rats plasma glucose was elevated, plasma insulin decreased, and enterocyte total and BBM levels of SGLT1 were increased, associated with diabetes induction. SFT drinking for 14 days in diabetic animals significantly decreased SGLT1 levels at the BBM but not in mucosa whole cell homogenates. This suggests effects of SFT on SGLT1 trafficking but not on total

protein levels. Because SFT reduced BBM SGLT1 levels in diabetic animals but had no effects in healthy controls, this plant extract seems to act only when the mechanisms of induction of BBM expression of intestinal glucose transporters are activated and does not affect basal expression levels.

To confirm the effect of SFT on the regulation of SGLT1 BBM levels, a model of its induced expression by dietary carbohydrate manipulation was used. As in the diabetic animals, SFT treatment significantly inhibited the increase of SGLT1 levels in the BBM after HC reintroduction, but not in whole cell homogenates. In an attempt to identify the active principle present in SFT, its main phenolic compound – RA – was used and shown to have an even stronger effect than the plant extract. The lower expression of SGLT1 in the BBM of RA-treated group was associated with a significant decrease of non-fasting plasma glucose levels measured in this group.

Our results indicate that SFT, and RA in particular, control the transporter levels at the BBM by decreasing it in conditions where it is enhanced, such as in diabetes and during adaptation to increased digestible carbohydrate in the diet. These effects on the trafficking of SGLT1 at intestinal level, are paralleled by decreases in blood glucose and identified RA as an active principle

There were no SFT effects on GLUT2 levels (total or BBM) that were only slightly increased (not in BBM) in diabetic animals. Intestinal BBM GLUT2 has been reported to be elevated in insulin resistant diabetic animals where the levels are not reduced by insulin treatment [28], contrary to levels of BBM SGLT1 that decrease upon insulin administration [32].

In agreement with previous reports [33], the intestinal level of Na^+/K^+-ATPase increased in association with diabetes. SFT treatment did not normalize these levels. Effects on the incretin hormone GLP-1 would also be important components of any

intestinal effects of SFT. The incretin hormone GLP-1 potentiates the glucose-induced insulin secretion by the beta-cells where it also has trophic effects [34, 35], and inhibits glucagon secretion, which justifies its clinical importance in the treatment of diabetes [36]. The number of GLP-1 expressing cells per cm of villus in diabetic animals was, however, not changed by SFT treatment.

The high antioxidant content of the sage extract [15] may contribute for the protection/regeneration of insulin-producing beta-cells. Antioxidants reduce oxidative stress and decrease glucotoxicity in pancreatic beta-cells, which have low antioxidant defenses [37]. There were, however, no changes in pancreatic beta-cell mass in diabetic animals after introduction of SFT treatment indicating no increased regeneration of the insulin producing cells by SFT.

In order to shed light on the mechanisms of *S. fruticosa* and RA on translocation to the BBM of SGLT1, heat shock protein 70 (Hsp70) and protein kinase C (PKC) were quantified. Hsp70 has been reported to increase both translocation of SGLT1 to the BBM and transport activity, through the formation of Hsp70/SGLT1 complexes [38, 39]. Our results show that Hsp70 levels profile in BBM was similar to that of SGLT1. This indicates that SGLT1 translocation and stabilization to the BBM may, in fact, be involved in the observed SFT effects.

PKC is implicated in the regulation of vesicle translocation containing glucose transporters such as GLUT4 and GLUT2 [40-42]. It is well established that insulin leads to the activation of atypical PKC and/or Akt (PKB), which results in the translocation of GLUT4 from intracellular pools to the plasma membrane of myocytes or adipocytes [41]. SGLT1 transporters reside also intracellularly in microtubule-associated vesicular structures, and respond to mechanisms of vesicle trafficking [43]. However, insulin signaling seems to have opposite effects in SGLT1 trafficking, since diabetic rats

treated with subcutaneous insulin significantly decreased BBM SGLT1 levels without changes in mRNA [32]. Our results show that although sage tea only slightly affected PKC levels in enterocytes, RA significantly decreased its expression. RA by decreasing PKC levels may be contributing to retain SGLT1 in intracellular stores. However, further research is needed to clarify the molecular mechanism(s) behind *S. fruticosa* and RA effects on SGLT1 levels in BBM, and the involvement and relationship of both Hsp70 and PKC in this process.

In conclusion, this study showed the ability of *S. fruticosa* to inhibit the adaptive increase of SGLT1 levels in BBM of rat enterocytes both after induction by STZ treatment and as a result of stimulation with HC diet. This effect appears to be due to modulation of SGLT1 trafficking by SFT, where RA seems to be the active principle. These data support previous reports on the antidiabetic effects of *S. fruticosa*, and emphasize the importance of the small intestine, and in particular the manipulation of BBM levels of SGLT1, in the therapeutic regulation of glucose homeostasis in diabetes.

Acknowledgements

We thank Dr. Hermann Koepsell for kindly supplying the SGLT1 antibody, Dr. Jane Dyer for providing us with protocol for brush-border membrane preparation and Dr. Karsten Kristiansen for reading the manuscript. We thank NANTA, Fábrica de Moagem do Marco S.A., Portugal for offering the Soybean meal 47.5 (LC diet). We also thank Alice Ramos from University of Minho for their help with animal handling.

MFA was supported by the Foundation for Science and Technology, Portugal, through the grant SFRH/BD/12527/2003. This work was supported by the Foundation for Science and Technology, Portugal, through the research grant POCI/AGR/62040/2004.

Conflicts of interest Statement

The authors state no conflict of interest.

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Figure captions

Figure 1. Schematic representation of experimental designs. **Experiment 1** (**A**): twenty-four rats were divided in to 4 groups of 6 animals each. STZ= streptozotocin; ip= intraperitoneal injection; Healthy= non-diabetic animals: Diabetic=STZ induced-diabetic animals. SFT treatment was from day 14^{th} until 28^{th} of experiment. Double vertical lines on the right side of each group indicate terminal sampling. ☐ healthy condition, ▨ developing diabetes and ■ SFT/water treatment with established diabetic condition. **Experiment 2** (**B**): thirty rats were divided into 5 groups with 6 animals each. Animals were fed either high-carbohydrate (HC) or low-carbohydrate (LC) diets and given either water (W), *S. fruticosa* tea (SFT), or rosmarinic acid (RA) to drink according to the schematic representation. Double vertical lines at the right end of each group indicate terminal sampling.

Figure 2. Effect of 14 days treatment with *Salvia fruticosa* tea (SFT) on fasting blood glucose (**A**), plasma insulin concentration (**B**) and liver glycogen content (**C**) of healthy (white bars) and STZ-induced diabetic (grey bars) animals. Values are means ± SEM, n=5-6. In **A**, diabetic rats showed high levels of blood glucose during the entire treatment period (from day 14^{th} until 28^{th}) when compared with healthy animals ($P \leq 0.001$); $^{**}P \leq 0.01$ when compared with diabetic water group by the Student t-test; NS, not significant ($P > 0.05$) when compared with the respective water group (Student t-test). In **B**, by two-way ANOVA, $^{+++}P \leq 0.001$ indicates that the difference between diabetic (W+SFT) and healthy (W+SFT) rats was significant; the post-hoc test Student Newman Keuls indicates significant differences: $^{***}P \leq 0.001$ and $^{**}P \leq 0.01$ when compared with the respective healthy control. In **C**, no statistically significant differences were obtained among treatments.

Figure 3. Western blot analysis of SGLT1 expression in jejunal total extract (**A**) and brush-border membrane (BBM) (**B**) of healthy (white bars) and STZ-induced diabetic rats (grey bars) treated with water (W) or SFT. Representative immunoblot on BBM fraction from two animals from each treatment group is present. Values are means ± SEM, *n*=5-6. Two-way ANOVA, indicates that the difference between diabetic (W+SFT) and healthy (W+SFT) rats was significant ($^{+++}P \leq 0.001$). The post-hoc test Student Newman Keuls indicates significant differences: $^{***}P \leq 0.001$, $^{**}P \leq 0.01$ and $^{*}P \leq 0.05$ when compared with the respective healthy control group, and $^{\#\#}P \leq 0.01$ when compared with the diabetic water group.

Figure 4. Western blot analysis of GLUT2 (**A**) and Na^+/K^+-ATPase (**B**) expression in jejunal whole cell homogenates of healthy (white bars) and STZ-induced diabetic rats (grey bars) treated with water (W) or SFT. Representative immunoblots of samples and corresponding loading control (beta-actin) from a pair of animals from each treatment group (**A'** and **B'**) are present. Values are means ± SEM, *n*=5-6. Two-way ANOVA indicates that the difference between diabetic (W+SFT) and healthy (W+SFT) was significant ($^{+}P \leq 0.05$ and $^{++}P \leq 0.01$). The post-hoc test Student-Newman-Keuls indicates significant differences: $^{**}P \leq 0.01$ when compared with the healthy water group.

Figure 5. Plasma glucose concentration of water drinking (W) animals maintained on a high carbohydrate diet (HC-HC; black bar), changed from a HC to low carbohydrate (LC) diet (HC-LC; grey bar), and finally returned to a HC diet following LC diet (HC-LC-HC; white bars). In addition to water drinking, HC-LC-HC animals were also given either sage tea (SFT) or rosmarinic acid (RA) *ad libitum* for the final 4 days on HC diet.

Values are means ± SEM, n= 6. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other (P>0.05). Effect of drinking on HC-LC-HC groups (white bars): $^{**}P \leq 0.01$ when compared with the HC-LC-HC/W group and $^{\#}P \leq 0.05$ when compared with HC-LC-HC/SFT group.

Figure 6. Expression levels of SGLT1 protein in jejunal total extract (**A**) and brush-border membrane (BBM) (**B**) from rats of the different treatment groups determined by Western blotting. Animals were feed either with their regular high carbohydrate (HC-HC; black bars), changed from a HC to low carbohydrate (LC) diet (HC-LC; grey bar) or returned to a HC diet following LC diet (HC-LC-HC; white bar). In this latter feeding regime animals were given either drinking water (W), *S. fruticosa* tea (SFT) or rosmarinic acid (RA) *ad libitum* for 4 days. Representative blots of samples from a pair of animals from each treatment group are present. Values are means ± SEM, n= 6. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other (P>0.05). Effect of drinking on HC-LC-HC groups (white bars): $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ when compared with the HC-LC-HC/W group.

Figure 7. Western blot analysis of Hsp70 expression in BBM of rats fed either their regular high carbohydrate (HC-HC; black bar), changed from a HC to low carbohydrate (LC) diet (HC-LC; grey bar) or returned to a HC diet following LC diet (HC-LC-HC; white bars). In this latter feeding regime animals were given either drinking water (W), *S. fruticosa* tea (SFT) or rosmarinic acid (RA) *ad libitum* for 4 days (**A**). Values are means ± SEM, n= 6. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other (P>0.05). Effect of drinking on HC-LC-HC groups (white bars): $^{*}P \leq 0.05$ when compared with the HC-LC-HC/W group. In (**B**), representative blot of samples from a pair of animals form each treatment group is

present. In (**C**), it is shown the difference on the expression levels of Hsp70 between whole cell homogenate (WCH) and BBM fraction from the same rat, loading 25 µg of protein each.

Figure 8. Western blot analysis of PKC expression in small intestine whole cell homogenates from rats fed either their regular high carbohydrate (HC-HC; black bar), changed from a HC to low carbohydrate (LC) diet (HC-LC; grey bar) or returned to a HC diet following LC diet (HC-LC-HC; white bars). In this latter feeding regime animals were given either drinking water (W), *S. fruticosa* tea (SFT) or rosmarinic acid (RA) *ad libitum* for 4 days (**A**). Representative blots of samples and corresponding loading control (beta-actin) from a pair of animals form each treatment group are present (**B**). Values are means ± SEM, $n = 6$. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other ($P>0.05$). Effect of drinking on HC-LC-HC groups (white bars): $^{*}P \leq 0.05$ when compared with the HC-LC-HC/W group.